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Dated 5 November 2003.

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PROVISIONAL SPECIFICATION

PHOSPHOPROTEIN PREPARATIONS FOR BIOACTIVE METAL ION DELIVERY AND TEETH REMINERALISATION

We,, PATRICK JOSEPH SILCOCK, a New Zealand citizen of 73 Ann Street, Dunedin, New Zealand, MARTIN MITCHELL FERGUSON, a British citizen of 342 Highgate, Dunedin, New Zealand, ADRIAN STEWART DENHAM KERR, a New Zealand citizen of 100 Litten Road, Howick, Auckland, New Zealand, JEAN-PIERRE GHISLAIN DUFOUR, a citizen of Belgium of 6 Royal Terrace, Dunedin, New Zealand and LYNTON ALEXANDER BRIDGER, a New Zealand citizen of 71 Pacific Parade, Army Bay, Hibiscus Coast, Auckland, New Zealand, do hereby declare this invention to be described in the following statement:

PHOSPHOPROTEIN PREPARATIONS FOR BIOACTIVE METAL ION DELIVERY AND TEETH REMINERALISATION

FIELD OF THE INVENTION

This invention relates to compositions and methods for delivering bioactive metal ions to humans and animals. It also particularly relates to compositions and methods for remineralising teeth and/or for preventing or treating dental caries and/or tooth erosion, dentinal hypersensitivity or gingivitis.

BACKGROUND OF THE INVENTION

Dental caries (or decay) and dental erosion are still widespread conditions, despite the fluoridation of the water supply in many countries and the use of fluoride toothpastes. Dental caries usually begins in the enamel of the tooth surface but may progressively destroy the hard tissues of the teeth. In many countries, about half of 5 year old children experience some tooth decay. In addition, some groups of people are, by virtue of their occupation, particularly susceptible to dental erosion and/or caries. For example, wine tasters and athletes such as elite cyclists who frequently sip on sports drinks, continually expose their teeth to low pH beverages which may cause the whole surface of the tooth to dissolve.

It is well known that dairy products have a protective effect against the development of dental caries. A number of investigations have suggested that it is primarily the protein component of dairy products, and casein in particular, that exerts an anticariogenic/remineralising action on tooth enamel. In addition, a particular fraction of active peptides in casein have been identified as being largely responsible for the anticariogenic/remineralising action. These are the calcium phosphate sequestering phosphopeptides, which constitute about 10% of the total weight of casein. These peptides contain a cluster of phosphoseryl residues [-Ser(P)-Ser(P)-Ser(P)-Glu-Glu] that markedly increase the solubility of calcium phosphate by forming colloidal casein phosphopeptide amorphous calcium phosphate complexes.

There are numerous patent publications directed to various compositions containing casein, caseinates, digests thereof or specific casein-derived phosphopeptides for use in caries inhibition and related dental applications.

For example, NZ patent specification 199891 describes toothpastes and dentifrices containing a caries and gingivitis inhibiting amount of casein, alpha-s-casein or phosvitin.

JP 59152317 describes an oral composition comprising mutanase (a drug for preventing tooth decay) together with casein, casein hydrolysate or a mixture thereof.

US 5833953, JP 9002928 and US 5427769 all describe various compositions for treating or preventing dental caries and containing micellar casein.

US 5130123 describes a dentifrice composition for inhibiting caries or gingivitis containing a water soluble salt of either a caseinate or a digest of a caseinate.

WO 82/03008 describes compositions for inhibiting caries and gingivitis, containing phosphoproteins or phosphopolypeptides containing a specified amino acid sequence, and in particular sodium caseinate, calcium caseinate or phosvitin.

JP 4077415 describes a dental calculus-preventing composition containing casein phosphopeptides in combination with a suitable excipient.

US 5015628 describes anticariogenic phosphopeptides having 5 to 30 amino acids and containing a specified amino acid sequence, and which may be obtained by tryptic digestion of casein.

WO 98/40406 describes specific calcium phosphopeptide complexes having anticaries efficacy. The phosphopeptides contain the Ser(P) cluster sequence motif [-Ser(P)-Ser(P)-Ser(P)-Glu-Glu] , and are said to be able to stabilize their own weight in amorphous calcium phosphate and amorphous calcium fluoride phosphate.

WO 00/06108 describes various formulations for the delivery of bioactive constituents to biological surfaces such as dental surfaces, comprising suspensions or solutions of one or more isolated and purified casein protein or salt thereof.

Compositions containing casein phosphopeptides have been reported as having superior anticaries/remineralising activity compared to compositions containing intact casein. However, the use of casein phosphopeptides has the disadvantage that digestion of casein (for example using the enzyme trypsin) to release the desired phosphopeptides also releases hydrophobic peptides which give the resulting digest a bitter flavour. This means that, for the product to have an acceptable flavour, fractionation of the digest to remove such hydrophobic peptides is required. In turn, this means that only a fraction of the casein-derived material is used; typically over 75% of the material is wasted.

The applicants have now surprisingly found that by partially hydrolyzing casein and subsequently partially cross-linking the partial hydrolysate, phosphoprotein preparations having superior calcium-binding and teeth remineralisation properties to those of unmodified casein or a partial casein hydrolysate can be obtained. Such phosphoprotein preparations have also been found to have an enhanced ability to bind other bioactive active metal ions.

JP 4-126039 describes a method of preparing a functional peptide by partially hydrolyzing a food protein, such as a protein obtained from soy beans, wheat or sweetcorn, or animal proteins such as gelatin, animal meat, fish meat or casein, followed by treatment of the resultant hydrolyzed peptide with transglutaminase or diluted acid. The resulting treated peptide is said to be free of bitterness. JP 4-126039 does not however describe the cation binding properties of such peptides, nor is a peptide obtained from casein specifically described therein.

WO 00/05972 and WO 01/0154512 describe chewing gum compositions containing casein or a modified casein such as polymerized hydrolyzed casein, as part of the elastomeric component of the gum. These publications do not however describe the cation binding properties of such modified casein.

It is an object of the present invention to provide methods and/or compositions useful for delivering bioactive metal ions, and methods and/or compositions for remineralising teeth, and/or preventing or treating dental caries, tooth erosion, dentinal hypersensitivity or gingivitis, which will go some way towards overcoming the disadvantages of the prior art, or at least provide the public with a useful choice.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a method of delivering a bioactive metal ion to a mammal, comprising administering to the mammal a composition comprising an effective amount of a source of the metal ion in combination with a phosphoprotein preparation, wherein the phosphoprotein preparation has been obtained by partially crosslinking a partial hydrolysate of casein or a caseinate.

In another aspect, the invention provides a composition for delivering a bioactive metal ion to a mammal, the composition comprising an effective amount of a source of the metal ion, a phosphoprotein preparation obtained by partially crosslinking a partial hydrolysate of casein or a caseinate, and one or more physiologically acceptable diluents or carriers.

In a further aspect, the invention provides the use, in the preparation of a composition for delivering a bioactive metal ion to a mammal, of a phosphoprotein preparation obtained by partially crosslinking a partial hydrolysate of casein or a caseinate.

Preferably, the metal ion is divalent.

In preferred embodiments, the metal ion is selected from the group consisting of calcium, iron, zinc, cobalt, copper and magnesium.

Preferably, the composition is an oral composition, in the form of a foodstuff or beverage, or a pharmaceutical vehicle such as a tablet or capsule.

Preferably, the pH of the composition is between about 6 and 9, more preferably between about 6.5 and 8, still more preferably between about 6.8 and 7.7, and most preferably between about 7 and 7.5.

In a further aspect, the present invention provides a method for remineralising tooth enamel and/or for treating or preventing dental caries, tooth erosion, dentinal hypersensitivity or gingivitis in a mammal, the method comprising contacting the teeth of the mammal with a composition comprising an effective amount of a phosphoprotein preparation, wherein the phosphoprotein preparation has been obtained by partially crosslinking a partial hydrolysate of casein or a caseinate.

In still a further aspect, the invention provides the use, in the preparation of a composition for remineralising tooth enamel and/or for treating or preventing dental caries, tooth erosion, dentinal hypersensitivity or gingivitis in a mammal, of a phosphoprotein preparation obtained by partially crosslinking a partial hydrolysate of casein or a caseinate.

In still a further aspect, the invention provides a composition for remineralising tooth enamel and/or for treating or preventing dental caries, tooth erosion, dentinal hypersensitivity or gingivitis in a mammal, wherein the composition comprises an effective amount of a phosphoprotein preparation in combination with one or more carriers or diluents, wherein the phosphoprotein preparation has been obtained by partially crosslinking a partial hydrolysate of casein or a caseinate.

In certain preferred embodiments, the composition further comprises a source of calcium ions.

Preferably, the composition also comprises a source of one or more of the following ions: phosphate, fluorophosphate and fluoride.

More preferably, the composition comprises both calcium and phosphate ions, conveniently added as calcium phosphate. Alternatively, the calcium and phosphate ions may be added as sodium phosphate and calcium chloride.

Preferably, calcium ions are present in the composition at a level of at least about 20 mmol calcium ions per gram of phosphoprotein preparation, more preferably at least about 30 mmol/g, and most preferably at least about 40 mmol/g.

Preferably, the molar ratio of calcium ions to phosphate ions is in the range of about 0.8-1.2:0.4-0.8, more preferably about 1:0.6.

In another alternate embodiment, the composition comprises a source of strontium ions. Preferably, in this embodiment the composition also comprises a source of fluoride ions.

In certain preferred embodiments, the composition is in the form of a foodstuff or confection, such as a chewing gum.

In alternative preferred embodiments, the composition is in the form of a mouthwash or a dentifrice, such as a liquid dentifrice, a toothpaste, a powder, an emulsion or a gel.

Preferably the partial hydrolysate is obtained by enzymatic hydrolysis of acid casein, rennet casein or a caseinate.

Preferably, the enzyme is selected from the group consisting of trypsin, pepsin, chymotrypsin and papain.

Most preferably, the enzyme is trypsin, conveniently porcine pancreatic trypsin.

Preferably, the partial hydrolysis is carried out at a pH of from about 7 to about 8.

Preferably, the molecular weight profile of the partially hydrolyzed casein or caseinate is less than that of casein but greater than the following distribution: about 1.7% ≥ 30,000Da, 22% < 30,000Da and ≥ 21,000Da, 22% < 21,000 Da and ≥ 12,000Da, 54.3% <12,000Da.

More preferably, the molecular weight profile of the partially hydrolyzed casein or caseinate is less than that of casein but greater than the following distribution: about 9.4% ≥ 30,000Da, 48% < 30,000Da and ≥ 21,000Da, 11% < 21,000 Da and ≥ 12,000Da, 31.6% <12,000Da.

More preferably, the molecular weight profile of the partially hydrolyzed casein or caseinate is less than that of casein but greater than the following distribution: about 11% ≥ 30,000Da, 50% < 30,000Da and ≥ 21,000Da, 10% < 21,000 Da and ≥ 12,000Da, 29% <12,000Da.

Most preferably, the molecular weight profile of the partially hydrolyzed casein or caseinate is less than that of casein but greater than the following distribution: about 13% ≥ 30,000Da, 53% < 30,000Da and ≥ 21,000Da, 8% < 21,000 Da and ≥ 12,000Da, 26% <12,000Da.

Preferably, the partial hydrolysate is partially crosslinked enzymatically, using either transglutaminase or lysyl oxidase, or using the plastein reaction.

Most preferably, the partial hydrolysate is partially crosslinked using the enzyme transglutaminase, preferably at a pH of from about 7 to about 8.

Preferably, the partial crosslinking is carried out under conditions sufficient to increase the amount of proteinaceous material in the partial hydrolysate having a molecular weight greater than about 30,000 Da by at least about 50%, more preferably by at least about 64%, even more preferably by at least about 80%, and most preferably by at least about 100%.

While the invention is broadly as defined above, it is not limited thereto and also includes embodiments of which the following description provides examples.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described in more detail and with reference to the accompanying drawings, in which:

Figure 1 shows the enamel remineralisation yield after treatment of acid etched tooth enamel with three different remineralising solutions, namely saliva, 10% caseinate solution, and 10% partially crosslinked hydrolyzed caseinate solution (as described in Example 3);

Figure 2 is a Scanning Electron Micrograph of the tooth enamel surface prior to acid etching;

Figure 3 is a Scanning Electron Micrograph of the tooth enamel surface after acid etching;

Figure 4 is a Scanning Electron Micrograph of the tooth enamel surface after both acid etching and treatment with polymerized hydrolyzed caseinate solution.

DESCRIPTION OF THE INVENTION

As defined above, the present invention relates to methods and compositions for delivering bioactive metal ions to mammals. It also relates to methods and compositions for remineralising tooth enamel and/or for treating or preventing dental caries, tooth erosion, dentinal hypersensitivity or gingivitis. In a related aspect, the invention also relates to novel phosphoprotein preparations useful in such methods and compositions.

The applicants have surprisingly found that by partially hydrolyzing casein and subsequently partially crosslinking the partial hydrolysate, a phosphoprotein preparation can be obtained that has superior cation binding properties to those of both unmodified casein and of the partial hydrolysate.

These properties make such phosphoprotein preparations useful as delivery vehicles for administering bioactive metal ions to humans or animals, particularly divalent cations including calcium, iron, zinc, cobalt, copper and magnesium. The phosphoprotein preparations are able to bind significant quantities of cations and thus prevent precipitation of and maintain salts of the cations in solution, thereby enhancing their bioavailability. Also, as the phosphoprotein preparations have been found to have a higher solubility than unmodified casein, they can be incorporated into oral compositions relatively easily.

The phosphoprotein preparations have particular application in remineralising tooth enamel and thereby in treating or preventing tooth erosion or dental caries. Without wishing to be bound by any theory, it is believed that the ability of the phosphoprotein preparations to solubilise calcium (and phosphate) ions may be at least partly responsible for their ability to remineralise tooth enamel. In particular, the phosphoprotein preparations are able to maintain a high concentration of calcium and phosphate ions in solution close to the tooth enamel surface, facilitating diffusion of these ions back into the pores of demineralised tooth enamel and thus increasing remineralisation activity. The phosphoprotein preparations have also been found to form a coating on the tooth enamel surface, which may further enhance the availability of calcium and phosphate ions.

The phosphoprotein preparations suitable for use in the invention may be obtained by partially crosslinking a partial hydrolysate of casein, that is, casein in which some, but not all, of the peptide bonds have been hydrolyzed.

The casein used to prepare the partial hydrolysate may be in any form; acid casein, rennet casein or a caseinate may all be used. Although chemical hydrolysis is by no means excluded, it is preferred that the partial hydrolysis is carried out enzymatically, in aqueous solution. Suitable enzymes for performing the partial hydrolysis include trypsin, pepsin, and chymotrypsin. Alternatively, a microbial protease may be used, such as proteases from *Aspergillus melleus* (optimum pH of 8, optimum temperature of 45°C) or *Bacillus subtilis* (optimum pH of 7, optimum temperature of 55°C). It is however particularly preferred that that the enzyme used is trypsin, conveniently porcine pancreatic trypsin.

The partial hydrolysis may be carried out at a temperature and pH appropriate to the enzyme being used. For example, if bovine derived trypsin is used, the partial hydrolysis may conveniently be carried out at a pH of about 7 to about 8, and at a temperature of about 37°C. It will be appreciated that at this pH, the casein will be present as a caseinate, eg sodium caseinate, depending on the buffer used in the reaction solution.

The reaction should be carried out for a sufficient period of time and under appropriate conditions, eg enzyme and casein concentrations, to allow the desired degree of hydrolysis to be achieved. When the desired degree of hydrolysis has been achieved, the reaction may conveniently be terminated by inactivating the enzyme, for example by heating the reaction mixture to a temperature which will denature the enzyme, eg about 80°C.

It is preferred that the partial hydrolysis is carried out under conditions which result in the partially hydrolyzed casein having a molecular weight profile that is less than that of casein but greater than the following distribution: about 1.7% ≥ 30,000Da, 22% < 30,000Da and ≥ 21,000Da, 22% < 21,000 Da and ≥ 12,000Da, 54.3% <12,000Da.

It is more preferred that the molecular weight profile of the partially hydrolyzed casein is less than that of casein but greater than the following distribution: about 9.4% ≥ 30,000Da, 48% < 30,000Da and ≥ 21,000Da, 11% < 21,000 Da and ≥ 12,000Da, 31.6% <12,000Da, and still more preferred that the molecular weight profile greater than the following distribution: about 11% ≥ 30,000Da, 50% < 30,000Da and ≥ 21,000Da, 10% < 21,000 Da and ≥ 12,000Da, 29% <12,000Da. It is most particularly preferred that the molecular weight profile of the partially hydrolyzed casein or caseinate is less than that of casein but greater than the following distribution: about 13% ≥ 30,000Da, 53% < 30,000Da and ≥ 21,000Da, 8% < 21,000 Da and ≥ 12,000Da, 26% <12,000Da.

The molecular weight profile of the partially hydrolyzed casein may conveniently be measured by size exclusion gel filtration, using techniques known to those persons skilled in the art. By way of example, the partially hydrolyzed casein may be dissolved in a suitable solvent,

conveniently 6M urea, and the protein fractions separated using fast protein liquid chromatography (FPLC system), for example using a Superdex 200 10/30HR column, and the eluted proteins detected by UV absorption (conveniently at 280 nm). The molecular weight distribution of the eluted proteins may then be calculated by integration of the protein absorption curve.

Those persons skilled in the art will appreciate that by varying the reaction conditions appropriately, such as the reaction time and enzyme concentration, a partially hydrolyzed casein having the desired molecular weight profile can be obtained. By way of example, a partially hydrolyzed casein having a particularly preferred molecular weight profile may be obtained by first solubilising a 10% isoelectric precipitated casein solution with NaOH to pH 7 at 50°C. The solution is then cooled to 37°C, and a porcine pancreatic trypsin preparation (a suitable preparation is commercially available as Novo.4500K, molecular weight 23,400 Da, activity 4500 USP units/mg) added at about 0.01% w/w casein and incubated for 15 minutes. Enzyme inactivation may be achieved by heating to 80°C and holding for 5 minutes.

Once the partially hydrolyzed casein has been prepared, it is then partially crosslinked to form a phosphoprotein preparation according to the invention.

The partial crosslinking may conveniently be carried out enzymatically, using either of the enzymes lysyl oxidase or transglutaminase.

It is particularly preferred that the enzyme transglutaminase is used, and that the polymerization is carried out at a pH between about 7 and 8. The partial crosslinking is desirably carried out under conditions and for a time sufficient to allow the desired degree of molecular weight increase to take place. Again, once the desired degree of crosslinking has been achieved, the reaction can be terminated by inactivation of the enzyme, typically by heating the reaction mixture to a temperature sufficient to denature the enzyme, for example to about 80°C for about 5 minutes. It is also generally preferred that, following completion of the partial crosslinking and deactivation of the enzyme, the resulting phosphoprotein-containing solution is dialyzed or diafiltered to remove any remaining low molecular weight peptides and salts, conveniently using

a membrane with a molecular weight cutoff of from about 10,000 to about 14,000 Da. The purified phosphoprotein-containing solution may be freeze dried or spray dried to obtain the phosphoprotein preparation in a solid form.

Any commercially available source of transglutaminase can be used to carry out the partial crosslinking. By way of example, a suitable enzyme is a 1% transglutaminase preparation commercially available from Ajinomoto Co. as Activa MP.

Although it is preferred that partial crosslinking of the partial casein hydrolysate is carried out enzymatically, partial crosslinking by chemical means, using a suitable reagent such as a bifunctional aldehyde (eg glutaraldehyde) is not excluded. Alternatively, the plastein reaction (which is known to those skilled in the art) may be used.

It is preferred that the partial crosslinking is carried out under conditions sufficient to increase the amount of proteinaceous material in the partial hydrolysate having a molecular weight greater than about 30,000 Da by at least about 50%, more preferably by at least about 64%, even more preferably by at least about 80%, and most preferably by at least about 100%.

Again, the molecular weight profile of the partially crosslinked partial hydrolysate may conveniently be measured using size exclusion gel filtration, as described above.

Those persons skilled in the art will appreciate that by varying the reaction conditions appropriately, such as the reaction time and enzyme concentration, a phosphoprotein preparation having the desired molecular weight profile can be obtained.

By way of example, a phosphoprotein preparation having a particularly preferred molecular weight profile, in which the amount of proteinaceous material in the partial hydrolysate having a molecular weight greater than about 30,000Da is increased by about 100%, may be obtained by treating a partially hydrolyzed casein prepared as described above with a transglutaminase preparation (Activa MP, commercially available from Ajinomoto Co.) added at a ratio of 4.5% w/w casein and incubating the reaction mixture at 40°C for 18 hours.

It has been found that phosphoprotein preparations obtained as described above and prepared from a partial casein hydrolysate in which the level of hydrolysis is relatively low and the subsequent level of partial crosslinking is controlled at a moderate level, are able to bind and thus solubilise particularly high levels of calcium ions and other bioactive cations.

The phosphoprotein preparations may be incorporated into compositions suitable for delivering bioactive metal ions, particularly divalent metal ions, to humans and animals. Such compositions may be in the form of pharmaceutical vehicles such as tablets or capsules. Tablets or capsules containing the phosphoprotein preparation, in combination with a source of a bioactive metal ion in a physiologically useful amount and one or more physiologically acceptable carriers or diluents, may be prepared using standard methods known to those skilled in the art. Alternatively, the phosphoprotein preparation may be incorporated into a foodstuff or beverage, in combination with a source of the bioactive metal ion it is desired to administer. For example, compositions containing the phosphoprotein preparations in combination with an effective amount of a source of calcium (such as calcium phosphate) or iron may be administered to humans or animals in need of calcium or iron supplementation, respectively.

The phosphoprotein preparations also have particular application in remineralising teeth, and in treating or preventing tooth erosion, dental caries, dentinal hypersensitivity or gingivitis. Compositions useful in such applications and suitable for contacting teeth with the phosphoprotein preparation may take a number of forms. For example, such compositions may take the form of a mouthwash or a dentifrice, such as a liquid dentifrice, a toothpaste, a powder, an emulsion or a gel containing the phosphoprotein. Alternatively, the phosphoprotein preparations may be incorporated into foodstuffs, such as confectionery or chewing gum.

It is preferred that, in addition to the phosphoprotein preparation, such compositions also contain a source of calcium ions, and preferably also a source of phosphate ions. For example, calcium phosphate may be included in the composition. Although calcium and phosphate ions are present in saliva, incorporating sources of these ions is preferred in order to take advantage of the calcium-binding properties of the phosphoprotein and maximize the concentration of calcium

and phosphate ions in contact with the tooth enamel. It is particularly preferred that calcium ions are present in the composition at a level of at least about 20 mmol calcium ions per gram of phosphoprotein preparation, more preferably at least about 30 mmol/g, and most preferably at least about 40 mmol/g. It is also preferred that the molar ratio of calcium ions to phosphate ions is in the range of about 0.8-1.2:0.4-0.8, more preferably about 1:0.6.

Other anions that may be included in the compositions include fluoride and fluorophosphate.

In other preferred embodiments, the compositions may include a source of strontium ions in addition to or instead of calcium ions. Such compositions may be particularly useful in treating dentinal hypersensitivity.

It is generally preferred that the pH of the compositions of the present invention, either in the form of compositions for delivering bioactive metal ions or for dental applications as described above, is buffered at a level between about 6 and 9, more preferably between about 6.5 and 8, still more preferably between about 6.8 and 7.7, and most preferably between about 7 and 7.5.

The invention will now be described in more detail with reference to the following examples.

EXAMPLES

Example 1 – Phosphoprotein preparation and determination of calcium-binding capacity

Tryptic hydrolysis

A 10 % isoelectric precipitated casein solution was solubilised with NaOH to pH 7.0 at 50°C. Once soluble, the solution was cooled to 37°C, and bovine derived trypsin (Novo) added at between 0.01 - 0.2 % w/w casein and incubated for up to 2 hours. Enzyme inactivation was achieved by heating to 80°C, and holding for 5 minutes.

Lot number	Enzyme concentration % weight/weight casein	Hydrolysis time /minutes
Lot 5	0.01	15
Lot 6	0.01	30
Lot 7	0.01	45
Lot 1	0.01	60
Lot 2	0.2	60

The molecular weight profiles of the phosphoprotein preparations were determined by gel filtration as follows. A 1% protein solution was prepared in 6M Urea, with 50mM sodium phosphate at pH 7.5 as the buffer. This solution was centrifuged at 10 000 x g for 10 minutes and passed through a 0.2μm filter. A sample volume of 500 μl injected into the 100μl sample loop of a Pharmacia FPLC fitted with a Superdex 200 10/30HR column. The running buffer used was 6 M Urea, with 50mM sodium phosphate at pH 7.5 and flow rate of 0.5 ml/min. Detection was by UV absorption (280 ηm). The protein absorption curve was integrated and arbitrarily divided into the following four molecular weight groupings:

1) greater than about 30,000 daltons
2) less than about 30,000 daltons and greater than about 21,000 daltons
3) less than about 21,000 daltons and greater than about 12,000 daltons
4) less than about 12,000 daltons.

Molecular weight profiles of Lots 5, 6, 7, 1 and 2 (after partial hydrolysis), expressed as % distribution of Lots 5, 6, 7, 1 and 2

Molecular weight range	**Lot number** 5	6	7	1	2
≥30,000	13.66	11.66	9.4	9.84	1.78
<30,000, ≥ 21,000	53.74	50.78	49.13	48.87	22.06
<21,000, ≥ 12,000	7.58	9.99	11.37	10.92	22.25
<12,000	25.02	27.57	30.1	30.37	53.91

Transglutaminase treatment

The pH was re-adjusted (as necessary) to 7.0, and transglutaminase (1% commercial preparation, Ajinomoto) added at a ratio of 4.5% w/w casein and incubated at 40°C for the desired length of time. Enzyme inactivation was achieved by heating to 80°C, and holding for 5 min. The modified protein solutions were freeze dried.

Molecular weight profiles of Lots 5, 6, 7, 1 and 2 (after treatment with transglutaminase), expressed as % distribution of Lots 5, 6, 7, 1 and 2

Lot 5

Molecular weight	**crosslinking time (hours)**		
range	**1**	**6**	**18**
≥30,000	26	38	27
<30,000, ≥ 21,000	44	26	19
<21,000, ≥ 12,000	7	13	15
<12,000	23	23	39
Total	100	100	100

Lot 6

Molecular weight	**crosslinking time (hours)**		
range	**1**	**6**	**18**
≥30,000	19	25	32
<30,000, ≥ 21,000	44	29	21
<21,000, ≥ 12,000	9	20	21
<12,000	27	25	26
Total	100	100	100

Lot 7

Molecular weight	crosslinking time (hours)		
range	**1**	**6**	**18**
≥30,000	16	22	28
<30,000, ≥ 21,000	44	30	22
<21,000, ≥ 12,000	11	21	21
<12,000	29	26	28
Total	100	100	100

Lot 1

Molecular weight	crosslinking time (hours)		
range	**1**	**6**	**18**
≥30,000	15	31	38
<30,000, ≥ 21,000	43	28	19
<21,000, ≥ 12,000	12	17	18
<12,000	30	24	26
Total	100	100	100

Lot 2

Molecular weight	crosslinking time (hours)		
range	**1**	**6**	**18**
≥30,000	1	2	2
<30,000, ≥ 21,000	23	21	25
<21,000, ≥ 12,000	27	29	28
<12,000	48	48	45
Total	100	100	100

Measurement of calcium binding capacity of the phosphoprotein preparations

The calcium binding capacity of the proteins was determined by re-suspending the protein in water, adding calcium and phosphate ions at a set ratio under constant pH; removing the

insoluble material (salts and protein); then removing the soluble non bound salts and determining the amount of calcium bound to the soluble protein. The experimental details were as follows.

A 1% solution of the proteins were dissolved with milli-Q water, and allowed to stand for 1 hour to ensure complete hydration. Calcium chloride was added at the following levels: 0mM, 10mM, 20mM, 30mM, 40mM and 50mM; and the solution incubated at 25°C for 1 hour with good mixing. Sodium phosphate was added at a molar ratio of 0.6 to the calcium. Throughout the experiment the pH was maintained at 7.0 using NaOH solution. The samples were incubated at 25°C for 6 to 10 hours with good mixing. After incubation, a sample was centrifuged at 10 000 x g for 10 minutes and filtered through a 0.2μm nylon filter and split into two portions.

One portion of the sample was injected into a 2ml sample loop and loaded onto a Pharmacia FPLC fitted with Sephadex G-25 (Vt=25 ml) desalting column. The running buffer was 10 mM HEPES at pH 7 the flow rate was 2ml/min and detection was achieved through UV absorption (280 ηm), conductivity and pH. The protein peak was collected and calcium concentration determined by atomic absorption spectroscopy (AAS).

The other portion was used to determine the soluble protein content as per the Folin protein assay.

Calcium binding capacity of Lot 5 before (0 hours) and after transglutaminase treatment (1, 6, or 18 hours) expressed as mg Ca^{2+} per g initial protein

mmol Ca^{2+} added	**Hours treated with transglutaminase**			
	0	**1**	**6**	**18**
0	0.0	0.0	0.0	0.0
10	n.d	n.d	n.d	n.d
20	n.d	n.d	n.d	n.d
30	3.2	7.3	24.2	31.2
40	1.1	1.2	34.7	37.6

Calcium binding capacity of Lot 6 before (0 hours) and after transglutaminase treatment (1, 6, or 18 hours) expressed as mg Ca^{2+} per g initial protein

mmol Ca^{2+} added	Hours treated with transglutaminase 0	1	6	18
0	0.0	0.0	0.0	0.0
10	21.7	n.d	20.8	18.6
20	37.9	37.1	40.7	39.8
30	9.3	32.3	32.1	40.2
40	0.0	2.6	7.1	4.2

Calcium binding capacity of Lot 7 before (0 hours) and after transglutaminase treatment (1, 6, or 18 hours) expressed as mg Ca^{2+} per g initial protein

mmol Ca^{2+} added	Hours treated with transglutaminase 0	1	6	18
0	0.0	0.0	0.0	0.0
10	18.3	23.6	23.2	22.2
20	n.d	32.6	34.3	41.1
30	16.1	24.6	28.5	32.0
40	3.0	2.4	0.0	7.4

Calcium binding capacity of Lot 1 before (0 hours) and after transglutaminase treatment (1, 6, or 18 hours) expressed as mg Ca^{2+} per g initial protein

mmol Ca^{2+} added	Hours treated with transglutaminase 0	1	6	18
0	3.3	0.5	0.3	0.0
10	n.d	17.9	16.2	18.4
20	29.5	33.4	34.7	38.0
30	24.6	16.0	21.9	36.4
40	2.4	1.9	6.1	21.1

Calcium binding capacity of Lot 2 before (0 hours) and after transglutaminase treatment (1, 6, or 18 hours) expressed as mg Ca^{2+} per g initial protein

mmol Ca^{2+} added	Hours treated with transglutaminase 0	1	6	18
0	0.0	0.0	0.0	0.0
10	23.2	16.7	16.6	15.1
20	41.3	n.d	37.9	n.d
30	7.2	8.8	7.0	7.8
40	4.2	n.d	2.7	4.7

Calcium binding capacity of caseinate expressed as mg Ca^{2+} per g initial protein

mmol Ca^{2+} added	mg Ca^{2+} bound
0	0.0
5	2.8
10	2.3
15	1.7

The different proteins described above represent a range of degrees of hydrolysis of casein (Lot 5 the least hydrolysed, Lot 2 the most), and a range of cross-linking of the hydrolysis products with transglutaminase (0 hours, no cross-linking and 18 hours the most crosslinked). Lot 5 proteins are the most resistant to Ca^{2t} induced precipitation with near maximum calcium loading 40 mg Ca/g protein still remaining soluble in a 40 mM Ca^{2+}solution. The 18 hour Tg crosslinked Lot 5 sample is the best phosphoprotein preparation to date.

With a greater degree of hydrolysis of the casein, the proteins, even after extensive Tg cross-linking, become less resistant to precipitation in the presence of higher Ca^{2+} concentrations. None of the non-crosslinked samples were resistant to precipitation, and resistance increased with greater cross-linking in all samples. The maximum amount of Ca^{2+} any of the proteins could bind was approximately 40 mg Ca^{2+} / g protein, and this is thought to be related to the amount of phosphoserine residues present in casein.

The ability of native casein to bind Ca^{2+} is demonstrated in the final table. It is rapidly precipitated with increasing Ca^{2+} concentrations and has a maximum binding of just 2.8 mg Ca^{2+}/ g casein at 5 mM Ca^{2+}.

Example 2 – Preparation of a phosphoprotein

Tryptic hydrolysis

A 10 % isoelectric precipitated casein solution was solubilised with NaOH to pH 7.0 at 50°C. Once soluble, the solution was cooled to 37°C, and porcine derived trypsin (Novo.4500K, molecular weight 23,400 Da, activity 4500 USP units/mg) added at 0.01% w/w casein and incubated for 15 minutes. Enzyme inactivation was achieved by heating to 80°C, and holding for 5 minutes.

Molecular weight profiles of Lot 5

Molecular weight range	
≥30,000	13.66
<30,000, ≥ 21,000	53.74
<21,000, ≥ 12,000	7.58
<12,000	25.02

Transglutaminase treatment

The pH was re-adjusted (as necessary) to 7.0, and transglutaminase (1% commercial preparation, Activa MP, Ajinomoto Co) added at a ratio of 4.5% w/w casein and incubated at 40°C for 18 hours. Enzyme inactivation was achieved by heating to 80°C, and holding for 5 min. The molecular weight material greater than 30,000 Da was increased by 100%.

Example 3 – teeth remineralisation (hardening)

The remineralising (rehardening) potential of three products was determined. The products were human saliva, caseinate solution and a phosphoprotein preparation obtained by partial crosslinking of partially hydrolyzed casein. The remineralising efficacy of the products was determined by measuring the recovery in hardness of the controlled acid etched human enamel following treatment.

Enamel preparation

The human unerupted third molars were used in all of the experiments. After extraction, the teeth were mechanically cleaned with anylac brush and deionised water, and stored until required in 10% buffered formalin solution (pH 7.0) at 4°C.

Before use, the teeth were thoroughly rinsed, cut longitudinally and then were embedded in epoxy resin (Araldite). Each specimen was hand-ground on a glass plate using silicon carbide grits, progressively of 240- to 600-grit, under running water. Fine polishing was achieved using the 8-inch Laps with 3-μm diamond abrasive for 5 minutes and with 1-μm diamond abrasive for another 5 minutes on a rotating polishing machine using distilled water to keep specimens moist. Between each polishing treatment, an ultrasonic bath was used for removing debris. The specimens were evaluated under a dissecting microscope (15x) and those with any evidence of cracks, flaws, developmental defects or extraction damage were rejected. The selected samples were stored until required in 10% buffered formalin solution (pH 7.0) at 4°C.

Enamel Demineralisation

The polished sound enamel specimens were individually demineralised in 25ml of 1% (w/v) citric acid solution for 10 minutes at 37°C to create eroded lesions. Following demineralisation, specimens were washed thoroughly with deionised water and stored in deionised water prior to the next step. The pH of the solution was 2.3.

Remineralisation

Preparation of the remineralisation products

Saliva: Submandibular saliva was collected from a healthy volunteer and 10 mmol/L sodium azide added. Twenty-five millilitres of saliva was used for each specimen in the remineralising procedure.

10% Caseinate Solution (CN-60Ca): CN-60Ca contained 36 mmol/L $(PO_4)^{3-}$ (at pH 9.0), 60 mmol/L Ca^{2+} and 10% (w/v) lactic acid casein. The solution was thoroughly mixed using a magnetic stirrer at room temperature. After incubation in a water bath at 50°C for 15 minutes and cooling to room temperature, 10 mmol/L sodium azide was added as a preservative and then adjusted to pH 7.5 with 10% (w/v) NaOH.

10% Phosphoprotein Solution (PC-60Ca): PC-60Ca consisted of 36 mmol/L $(PO_4)^{3-}$ (at pH 9.0), 60 mmol/L Ca^{2+} and 10% (w/v) phosphoprotein preparation obtained by partial crosslinking of partially hydrolyzed caseinate (PC). The phosphoprotein preparation was obtained by treating a caseinate solution having a degree of hydrolysis of approximately 4% with the enzyme transglutaminase, as described in Example 2. Other conditions were as described for CN-60Ca7.5.

Protocol for Enamel Remineralisation

The selected specimens were treated in separate remineralisation solutions. The specimens were immersed in 40ml of fresh remineralisation solution (under constant shaking) for four hour time periods at 37°C in individual sealed 80-ml beakers. The CN60Ca was also incubated for a 16 hour time period.

Evaluation of Demineralisation/Remineralisation

Surface Microhardness (SMH) Evaluation

The surface microhardness (SMH) of the enamel blocks were measured with a Leitz MiniLoad-Hardness instrument using a Vickers diamond under a 200g load for 20 seconds. The specimens

were held perpendicular to the indentor to the compound stage of the hardness instrument. Fifteen indentations were averaged on each surface of the individual specimens for surface hardness determinations (after standardisation of the diamond indenter and using the 400x magnification). A distance of at least two times the indentation lengths for enamel was kept between the indentations to minimise interactions between neighbouring indentations. The SMH readings were taken at three stages of enamel demineralisation/remineralisation model as follows: (1) before exposure to the demineralisation solution (DS), (2) after exposure to the DS (1% citric acid) and softening and (3) after exposure to remineralisation solution or control solution. Following the initial examination of SMH (stage 1), each specimen underwent 10 minutes of demineralisation at 37°C in DS. Each specimen was immersed individually in 25 ml of solution. After exposure, the specimens were then removed from the solution, washed thoroughly in deionised water, blotted dry with 3mm filter paper and taken for SMH re-examination (stage 2). After the selected remineralisation treatment, the specimens were removed from the solution, thoroughly washed in deionised water, blotted dry again and re-tested for SMH (stage 3). The softening and rehardening potentials of the DS and the different remineralisation solutions were calculated for each specimen by subtracting the baseline SMH value (stage 1) and rehardening value (stage 3), respectively, from the softening value (stages 2).

The relationship between the measured SMH and the length of the Vickers indent average of the two diagonals was determined by equation 1:

$$SMH\ (kg/mm2) = 1854 \times P / d2 \qquad [1]$$

where P is the load in grams and d is the average length of the diagonals of the indentation measured in microns. The SMH numbers are directly from the microscopic measurements with Leitz MiniLoad-Hardness instrument used in the present study. In order to compare quantitatively the SMH numbers for the de- and remineralised specimens on a linear scale, it was necessary to convert the pre- and post-SMH measurements to values proportional to the yield of change between softened SMH and the amount of remineralisation achieved. For comparison between these two results, the difference of the remineralising effect was calculated using equation 2:

$R\ (\%) = [(PSMH)3 - (PSMH)2] / [100 - (PSMH)2]$ [2]

where PSMH3 and PSMH2 mean as follow:

PSMH3 = [SMH of stage 3/SMH of stage 1] x 100%
PSMH2 = [SMH of stage 2/SMH of stage 1] x 100%

Therefore *R* in equation 2 is the rehardening yield, remineralisation yield, or recovery yield, of stage 3 in this study.

Results

Table 1. Effect of submandibular saliva, caseinate solution and phosphoprotein preparation (obtained by partial crosslinking of partially hydrolysed casein) on the etched enamel surface

Specimen No	Surface Microhardness (kg/mm^2) Stage 1 Mean (SD)	Stage 2* Mean (SD)	Stage 3 Mean (SD)	
Saliva for 4 hours:				
Saliva 1	398.6 (21.8)	331.4 (34.3)	380.3 (30.5)	**
Saliva 2	370.3 (13.6)	285.0 (35.3)	349.7 (22.3)	**
Saliva 3	405.0 (27.7)	290.3 (26.4)	352.6 (20.8)	**
Saliva 4	379.6 (14.9)	293.2 (25.6)	344.3 (17.8)	**
CN-60Ca for 4 hours:				
CN 4	350.6 (30.4)	281.9 (15.4)	279.1 (20.3)	***
CN 5	405.9 (29.5)	319.7 (20.3)	314.0 (23.2)	***
CN 6	370.4 (19.1)	282.3 (26.7)	293.0 (26.6)	***
CN 7	397.2 (18.6)	313.4 (19.5)	316.0 (15.8)	***
CN-60Ca for 16 hours:				
CN 8	394.5 (19.0)	293.4 (33.3)	339.1 (26.4)	**
CN 9	422.5 (24.5)	339.8 (23.7)	325.1 (20.1)	***
CN 10	376.5 (30.4)	324.2 (30.7)	362.4 (30.5)	(p=0.003)
CN 11	387.0 (20.4)	315.8 (30.0)	335.6 (20.5)	(p=0.015)
PC-60Ca for 4 hours				
PC-60Ca 1	356.9 (20.5)	307.0 (23.2)	338.5 (36.9)	**
PC-60Ca 2	411.7 (19.5)	296.9 (22.5)	336.7 (35.4)	**
PC-60Ca 3	378.0 (20.4)	300.4 (15.1)	384.9 (16.1)	**
PC-60Ca 4	374.9 (14.7)	281.2 (19.5)	394.9 (25.0)	**
PC-60Ca 5	379.6 (13.9)	286.6 (19.5)	400.3 (28.9)	**
PC-60Ca 6	365.2 (11.8)	293.5 (26.3)	364.3 (18.3)	**
PC-60Ca 7	374.9 (30.3)	324.4 (30.5)	364.9 (30.6)	**
PC-60Ca 8	405.1 (21.3)	336.7 (19.8)	406.5 (37.1)	**
PC-60Ca 9	388.1 (21.9)	280.0 (36.2)	396.7 (22.8)	**
PC-60Ca 10	399.5 (26.3)	289.8 (09.1)	369.3 (17.0)	**
Control groups:				
PC-noCa 1	347.6 (19.2)	269.4 (13.7)	277.4 (20.5)	***
PC-noCa 2	367.7 (18.7)	302.4 (20.6)	307.5 (25.7)	***

noPC-60Ca 1	362.5 (18.9)	299.4 (20.6)	278.7 (18.7)	***
noPC-Ca 2	373.0 (21.0)	294.1 (22.2)	291.0 (31.4)	***

N = 15 (each specimen).

Stage 1: Microhardness testing in initial stage.

Stage 2: Microhardness testing after 1.0% citric acid etching for 10 minutes.

Stage 3: Treatment group: Microhardness testing after treatment with remineralising solution.

* All stage 2 values were significantly less than stage 1 values ($P < 0.001$).

** Stage 3 values were significantly greater than stage 2 values in treatment group ($P < 0.001$).

*** Stage 3 values were not significantly greater than stage 2 values in control group.

The Effect of Saliva on Etched Enamel Surface

Table 1 shows that human submandibular saliva significantly increased the SMH numbers of etched enamel after the four-hour treatment. The relative SMH after acid etching (Stage 2) was decreased to 77.3% of its original value and after treatment with the saliva this increased to 91.9% (Stage 3). The mean of the remineralisation yield (*R*) was 65.5%.

The Effect of 10% Caseinate (CN) on Etched Enamel Surface

It required 16 hours treatment with 10% caseinate solution (CN-60Ca7.5) before significant rehardening was found; there were no effects observed in the 4-hour groups. After treatment for 16 hours, SMH numbers significantly increased in three out of the four specimens ($P = 0.001$, $P < 0.003$, $P = 0.015$, respectively) (Table 1). The relative SMH after acid etching (Stage 2) was decreased to 80.6% of its original value and after treatment with the caseinate solution for 16 hours this increased to 86.5% (Stage 3). The mean *R* was 32.1%.

The Effect of Phosphoprotein obtained by Partial Crosslinking of Partially Hydrolyzed Casein (PC) on Etched Enamel Surface

Table 1 shows the change of SMH numbers of enamel surface after treatment with the phosphoprotein preparation obtained by partial crosslinking of partially hydrolyzed casein (PC) with 36 mmol/L $(PO_4)^{3-}$, 60 mmol/L Ca^{2+} (PC-60Ca). The SMH numbers of etched-enamel were significantly increased after the 4-hours incubation in PC-60Ca with all ten specimens. Seven out of ten specimens almost returned to the initial (before-etching) SMH values. The SMH numbers

were significantly higher than that of initial level in two tests (20%) (P = 0.012) after treatment with PC-60Ca.

The relative SMH after acid etching (Stage 2) was decreased to 78.3% of its original value and after treatment with the PC solution for four hours this increased to 98.2% (Stage 3). The remineralisation yield (*R*) was 92.1% (n = 10).

The exposure of acid etched enamel to the phosphoprotein solution without Ca^{2+} (PC-noCa) or to calcium phosphate buffer with no phosphoprotein preparation (no PC-60Ca) resulted in no significant regain in hardness (Table 1).

Summary of the performance of the different remineralisation solutions on the recovery yield of the etched enamel surface

The enamel remineralisation yield is presented in Figure 1. The difference in remineralisation yield between solutions was clear. PC-60Ca produced distinctly greater remineralisation potential than that of others.

De- and Remineralisation Assessment by Scanning Electron Microscopy (SEM)

Examination of the enamel surface by Scanning Electron Microscopy prior to etching showed the surface to be smooth (Figure 2). In contrast, it was found that acid etching of sound enamel resulted in (1) a loss of surface enamel, (2) an increase in the tooth surface area due to the roughening of the tooth surface and (3) exposure of a more reactive surface following the removal of superficial inert enamel (Figure 3).

After treatment with PC-60Ca, the etched enamel surface was covered with a relative smooth and dense coating with frequent rod-shaped products. In most areas, a moderately uniform surface coating was present with adherent reaction products of 0.5-1 μm in length. The coating was sufficiently dense to obscure the enamel prisms. The distribution of the deposits was relatively homogeneous and the deposits covered almost all the enamel surface (Figure 4). The presence of small rod-shaped products was assumed to be phosphoprotein-calcium phosphate complexes (PCCPC), though morphologic appearance alone does not identify a chemical

compound. It can be seen that many rod-shaped products were present in the demineralised interprismatic regions.

Discussion

Exposure of demineralised enamel surface to remineralisation solutions showed a regain in microhardness. This may indicate a partial restoration of the calcium phosphate content. The net result was a filling up of intra- and interprismatic spaces, which was assessed directly by SEM morphological observation and indirectly by indentation length measurements. A reduced porosity of the enamel surface in the SEM causes an increased resistance to the indenter penetration into the test surface, which is reflected by a smaller indentation length and suggests that remineralisation has occurred.

Remineralisation Effect of Saliva on Etched Enamel Surface

That saliva resulted in the rehardening of the etched enamel was expected as the remineralising ability of saliva is well reported (Koulourides *et al.* 1965, Leach *et al.* 1989, Peretz *et al.*, 1990). Saliva can be described as "the bloodstream of the teeth" - being rich in minerals and proteins and supersaturated with respect to calcium and phosphate ions. It surrounds and bathes the tooth and provides a constant supply of ions to the enamel surface (Peretz *et al.*, 1990). When cleaned enamel is wet by saliva, specific proteins (such as statherins and proline-rich proteins) from the saliva are adsorbed onto the teeth surface to form the salivary pellicle or acquired pellicle. These two protein groups are thought to inhibit primary (spontaneous) and/or secondary precipitation (crystal growth) of calcium and phosphate from saliva. This appears to be a necessary and important activity in the oral cavity because human saliva is supersaturated with respect to most calcium phosphate salts. These precipitation inhibitors keep the saliva in a state of supersaturation. The pellicle plays an important role in protecting the enamel by serving as a diffusion barrier.

An etching or carious lesion occurs in particular locations on the enamel surface where the equilibrium is upset and where a net loss of mineral has occurred. The physical nature of the

surface enamel, the saliva and the acquired pellicle can be considered as analogous to defence mechanisms present in other systems in the body.

Remineralisation Effect of Phosphoproteins obtained by Partial Crosslinking of Partially Hydrolysed Casein (PC) and Caseinate on Etched Enamel Surface

Under the experimental conditions, PC and caseinate were shown to coat on, incorporate in and reharden the etched enamel surface when assessed by microhardness testing and SEM. The SMH numbers of etched enamel showed almost complete recovery after treatment with the PC solution. The SEM showed a dense coating layer was precipitated on the enamel surface that resisted removal, even after 10 minutes of water washing. However, the crystalline nature of the surface enamel cannot be determined by the SEM method used in the present study.

The caseinate was not as effective as the PC and required a treatment time of 16 hours to achieve significant rehardening. The major reason for this was believed to be the low solubility of the caseinate in the presence of calcium and phosphate ions.

For successful rehardening of the enamel when using the PC calcium and phosphate ions had to be present. The omission of any one of the three components (phosphoprotein, calcium and phosphate) resulted in no remineralisation phenomenon being observed. Although the time period and chemical conditions (the conditions will be more complex in oral environment) are empirical, the key conclusion is that the de- and remineralisation occurred in this model system.

The possible reasons for the high remineralising ability of the phosphoprotein in conjunction with the calcium and phosphate ions are as follows:

1) The hydrolysis process followed by the partial crosslinking process results in a protein with greater stability than that of caseinate. The hydrolysis treatment followed by the partial crosslinking treatment appears to interfere with the self-associating nature of casein and appears to inhibit its micelle-forming tendency. Due to this decreased tendency to self associate the protein will remain soluble in the presence of high levels of calcium and phosphate.

2) The calcium phosphate rich phosphoprotein is the main component of the PC solution. The promotion of enamel remineralisation by the PC is consistent with the protein solubilising calcium and phosphate ions being at least partially responsible for remineralisation activity of casein. It is thought that the PC increased the solubility of calcium ions in solution, resulting in a higher concentration of free calcium ions available into the pores of etched enamel surface to facilitate remineralisation activity.

3) Without wishing to be bound by any theory, the remineralisation model of the phosphoprotein obtained by partial crosslinking of partially hydrolysed casein may be related to its ability to coat on and incorporate in the etched enamel surface. The PC contains closely situated groups of phosphoseryl residues that bind calcium phosphate and hydroxyapatite very strongly. These sections of the protein could calcium-bridge, ionically interact, and hydrogen-bind with the enamel surface. On binding to the hydroxyapatite surface, the protein coating may act as a reservoir, releasing calcium ions available for use in remineralisation or redeposition into areas of demineralisation in the crystal lattice. As shown in the present study, casein phosphoprotein-calcium phosphate particles are present on the enamel surface of the calcium-releasing bonding systems, forming a potential protective deposit on the enamel surface. Dissolution of calcium ions from casein phosphoprotein-calcium phosphate complexes (CPPCPC) and diffusion into the pores in the enamel may have occurred.

Example 4

Measurement of iron binding capacity of the phosphoprotein preparations

The iron binding capacity of the proteins was determined by re-suspending the protein in water, adding $FeCl_2$ under constant pH; removing the insoluble material (salts and protein); then removing the soluble non bound salts and determining the amount of calcium bound to the soluble protein. The experimental details were as follows.

A 1% solution of the proteins were dissolved with milli-Q water, and allowed to stand for 1 hour to ensure complete hydration. Ferrous chloride was added at the following levels: 0mM, 10mM, 30mM, and 40mM; and the solution incubated at 25°C for 1 hour with good mixing. Throughout the experiment the pH was maintained at 7.0 using NaOH solution. The samples were incubated

at 25°C for 6 to 10 hours with good mixing. After incubation, a sample was centrifuged at 10 000 x g for 10 minutes and filtered through a 0.2μm nylon filter.

The sample was injected into a 2ml sample loop and loaded onto a Pharmacia FPLC fitted with Sephadex G-25 (Vt=25 ml) desalting column. The running buffer was 10 mM HEPES at pH 7 the flow rate was 2ml/min and detection was achieved through UV absorption (280 ηm), conductivity and pH. The protein peak was collected and iron concentration determined by atomic absorption spectroscopy (AAS).

Iron binding capacity of Lot 6 after cross-linking with Tg for 18 hours. Expressed as mg Fe^{2+}/g initial protein, and mg Fe^{2+}/g of soluble protein.

[Fe^{2+}]	mg Fe^{2+}/g protein (total)	mg Fe^{2+}/g protein (soluble)
0 mM	0.0	0.0
10 mM	2.9	6.4
30 mM	3.5	13.8
40 mM	3.7	19.5

Fe^{2+} was found to bind to the Lot 6, 18 hr Tg phosphoprotein preparation at a maximum of 3.7 mg Fe^{2+}/g protein. Of the protein that remained soluble, the Fe^{2+} bound at about 20 mg Fe^{2+}/g soluble protein.

Example 5

Teeth remineralising emulsion

Ingredients	**% (w/w)**
Acacia gum	17.45
Sorbitol	4.5
GMS 400V	1
Glycerol	1

Maltitol syrup	7.5
Medium chain triglycerides	20
Water	46.9
Methyl Paraben	0.025
Propyl Paraben	0.075
Phosphoprotein (Example 2 - Lot 5)	1
$CaCl_2.2H_2O$	0.27
NaH_2PO_3	0.28
Peppermint oil	0.037

Formulation

1. Mix water and Maltitol syrup. Blend Acacia gum, protein, calcium chloride, sorbitol, methyl paraben and propyl paraben and disperse in water. Once the above powders have solubilised/dispersed add the NaH_2PO_3.

2. Heat GMS and glycerol until molten, add Medium chain triglycerides and re-heat solution to remove lumps (about 65°C).

3. Heat gum-protein solution to similar temperature as the GMS - Medium chain triglycerides mix. Blend both mixes together to get lump-free coarse emulsion.

4. Adjust solution pH with 3M NaOH to pH 7.

5. Add peppermint flavour.

6. Heat emulsion to 90°C.

7. Homogenise with Ultraturrex to obtain fine stable emulsion.

8. Rinse clean bottle with 96% ethanol, add hot emulsion to clean bottle.

Example 6 – Calcium burst film

Ingredients	%weight/weight
Phosphoprotein preparation (Example 1, Lot 6, 16 hours treatment with transglutaminase)	15.00
Glycerol	5.00
Peppermint	2.00
DATEM (emulsifier)	0.25
Milk-derived calcium phosphate	1.00
Water	76.75

The glycerol, peppermint and emulsifier were mixed together then added to the water and blended to achieve a coarse emulsion. The phosphoprotein was blended with the milk-derived calcium phosphate and added to the above solution with high intensive mixing and the pH adjusted to 7.0. The solution was heated to 70°C, emulsified then degassed. The degassed protein solution was cast on a TLC plate spreader achieving a 0.5 mm initial thickness. The film was dried in an oven at 100°C for 5 minutes.

INDUSTRIAL APPLICATION

It is believed that the methods and compositions of the present invention will find application in delivering bioactive metal ions, such as calcium and iron, to mammals. The invention is also expected to find particular application in compositions for remineralising teeth and/or for preventing or treating dental caries, tooth erosion, dentinal sensitivity or gingivitis.

The present invention is believed to possess certain advantages over teeth remineralising/anticaries compositions comprising phosphopeptides obtained from partial hydrolysis of casein, such as those described in US 5015628. In particular, the phosphoprotein preparations used in the present invention have a clean flavour, in contrast to partial casein hydrolysates, which contain bitter-tasting hydrophobic peptides that need to be removed in order

the product to have an acceptable flavour. In addition, the phosphoprotein preparations of the present invention utilize the vast majority of the proteinaceous material from the casein (rather than just the proportion containing the casein phosphopeptides), thereby reducing wastage.

The phosphoprotein preparations used in the present invention also have advantages over remineralising/anticaries compositions containing unmodified casein or caseinate; in particular, the calcium-binding and teeth remineralising properties of the phosphoprotein preparations are, at least in the preferred embodiments, significantly superior to those of casein. In addition, the phosphoprotein preparations are relatively soluble and have a lower viscosity than unmodified casein, thereby facilitating their incorporation into compositions.

Although the present invention has been described with reference to particular embodiments, those persons skilled in the art will appreciate that numerous variations and modifications may be made without departing from the scope of the invention.

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Figure 1

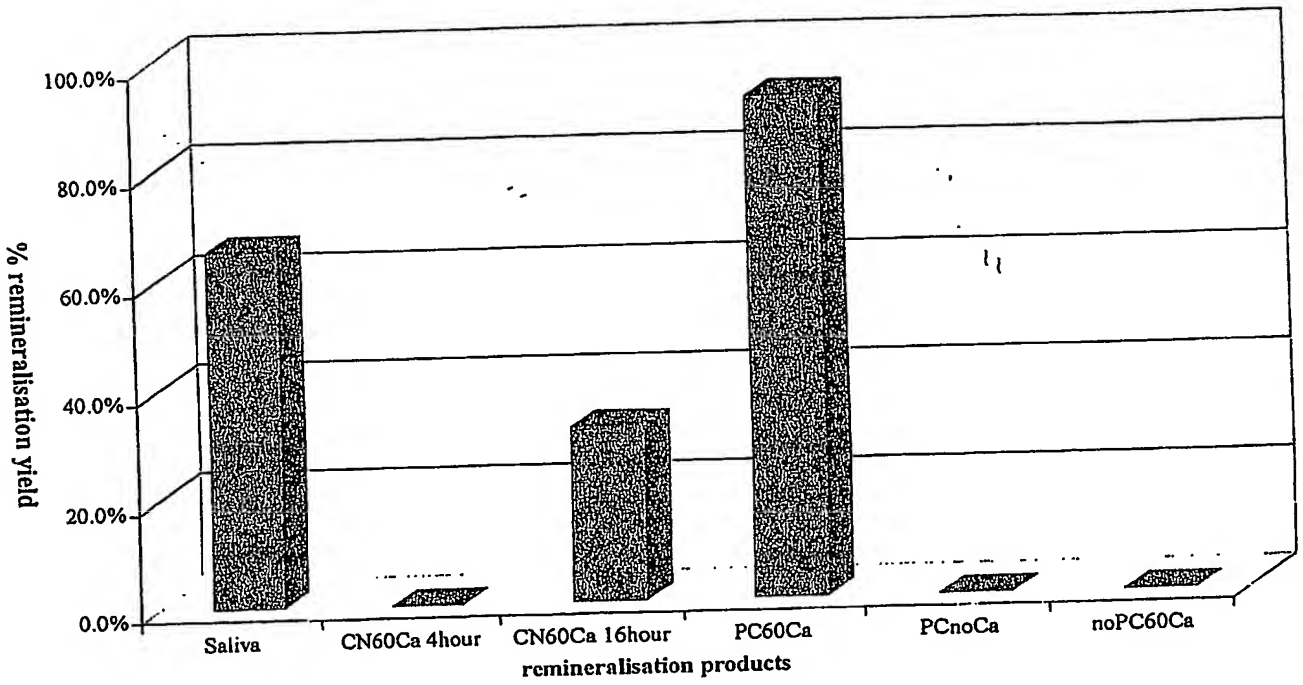

100.0%
80.0%
60.0%
40.0%
20.0%
0.0%
% remineralisation yield
Saliva
CN60Ca 4hour
CN60Ca 16hour
PC60Ca
PCnoCa
noPC60Ca
remineralisation products

Figure 2

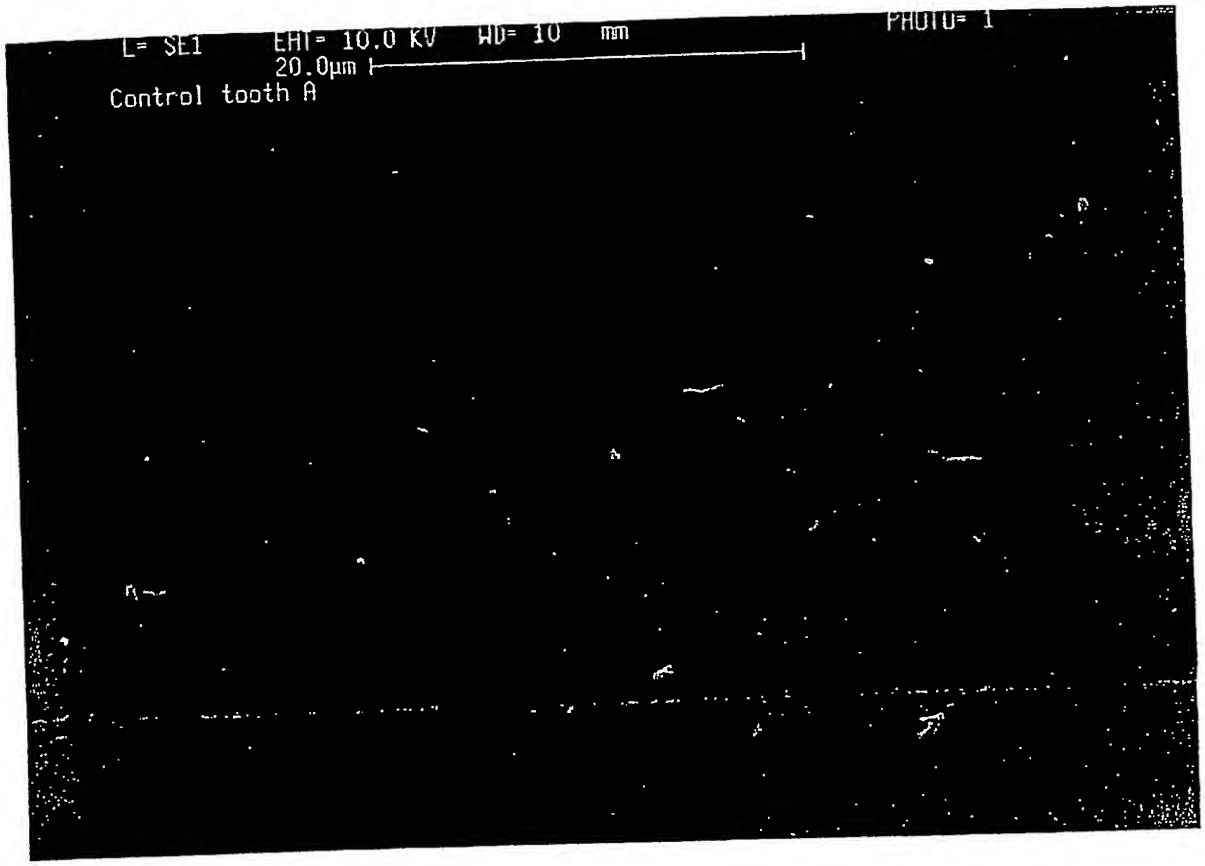


Figure 3

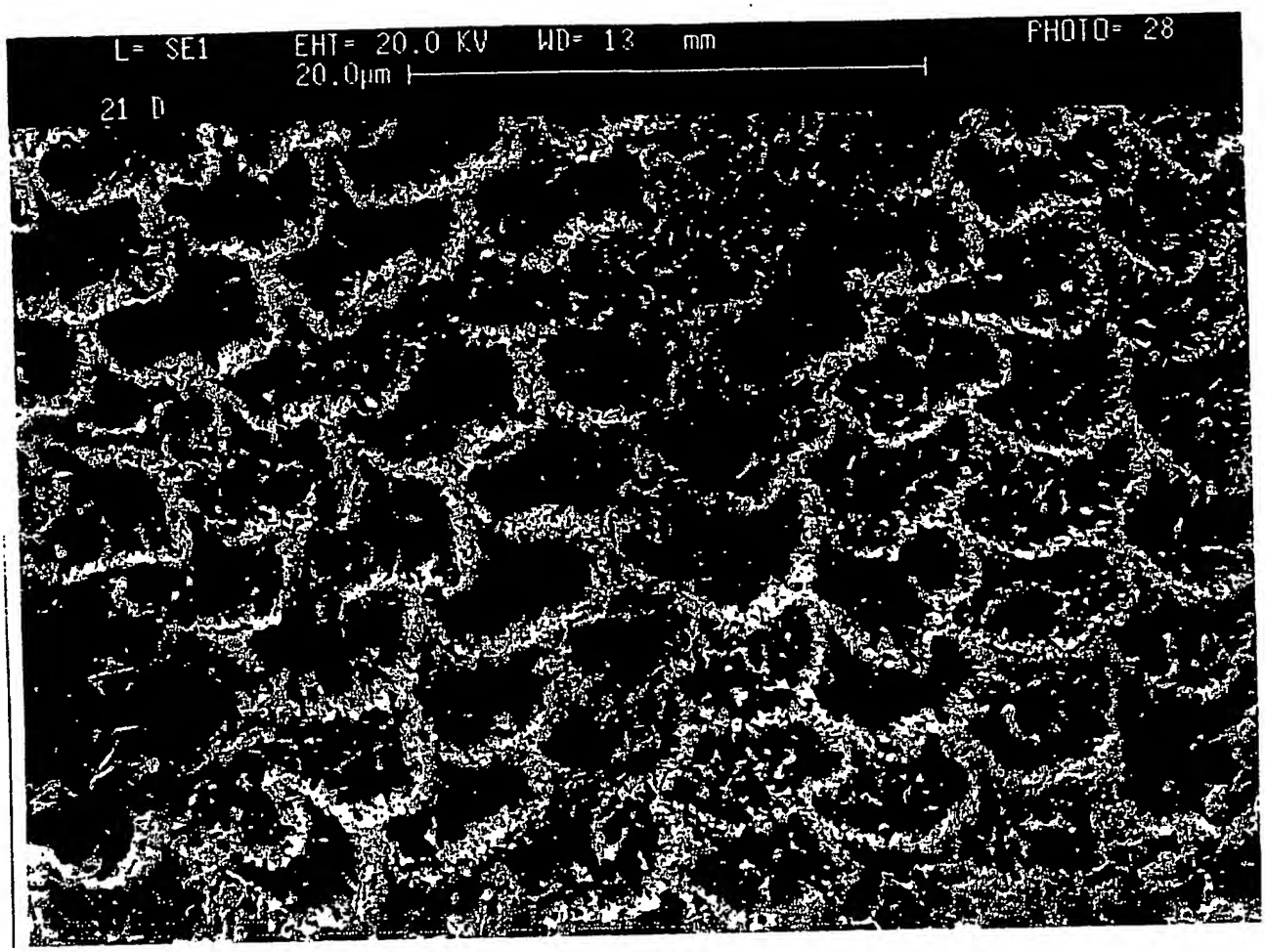
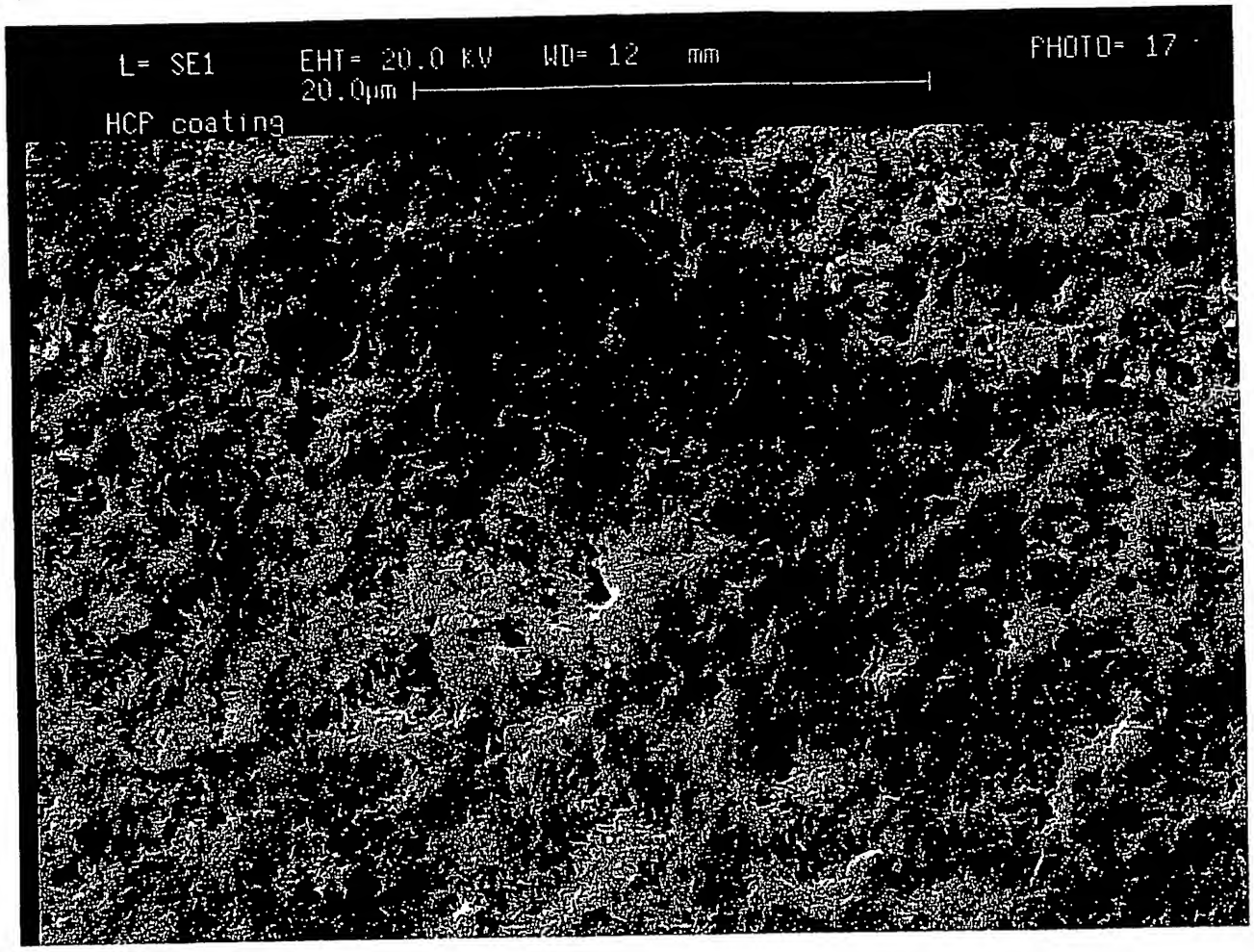


Figure 4



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